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THE GASES OF SWAMP RICE SOILS

PART V. A METHANE-OXIDIZING BACTERIUM FROM RICE SOILS

BY

P. A. SUBRAMANIA AIYER, B. A.

Assistant to the Government Agricultural Chemist, Madras

PART VI. CARBON DIOXIDE AND HYDROGEN IN RELATION TO RICE SOILS

BY

W. H. HARRISON, D. Sc.

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THE GASES OF SWAMP RICE SOILS.

PART V.

A METHANE-OXIDIZING BACTERIUM FROM RICE SOILS.

BY

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[Received for publication on 8th October, 1918.]

THE investigation of the gases of swamp rice soils has shown that the organized film overlying the surface of the soil is capable of oxidizing methane to carbon dioxide and water[1] and that this reaction is brought about by the agency of bacteria. An energetic oxidation of the gas was developed in mixed cultures of bacteria, but all attempts to isolate a pure species by following the methods of previous workers failed, and this fact led to the conclusion that the oxidation in these rice soils is due either to bacteria acting together in association or to a species distinct from the *B. Methanicus* of Söhngen.[2] Consequently the reaction was deemed worthy of closer investigation, particularly as Söhngen has shown in subsequent communications that *B. Pyocyanium* and other species possess the power of oxidizing methane and that *B. Pyocyanium*, *B. Fluorescens* and *M. Paraffinæ* can oxidize such substances as benzene and kerosine. Stormer also states that *B. Hexacarbovorum* is capable of causing the oxidation in question.

The crude cultures referred to above develop readily in purely mineral culture liquids[3] and when plated out in washed agar containing the same salts numerous colonies develop, but in no single instance were they found to possess the power of oxidizing methane. Attention was then turned to the use of silica jelly as a medium with the result that a pure species was isolated and studied.

[1] Harrison and Subramania Aiyer. *Mem. Dep. Agri., India, Chemical Series*, Vol. IV, No. 1.
[2] Söhngen. *Centl. Bakt.*, II Abt., XV (1905), p. 573.
[3] Harrison and Subramania Aiyer. *loc. cit.*

Most of the usual methods of preparing silica jelly were employed, but as a whole they were found to be either cumbersome or unreliable, and it was not until a modification of Stevens and Temple's[1] method was adopted that success was readily attained. This method of preparing the medium was found to be easy and reliable and consequently it is described in full.

Preparation of silica jelly and method of isolation.

The method of Stevens and Temple produces a solution of silica which contains from 12 to 20 parts of sodium chloride (NaCl) per 1,000, but this concentration was found to exercise an inhibitory effect on the growth of the specific bacterium and any attempt to remove it by dialysis resulted in removing at the same time the traces of hydrochloric acid (HCl) which are necessary to prevent the solution from coagulating. This difficulty was obviated by carrying on the process of dialysis by means of distilled water containing 2—4 parts of $\frac{N}{2}$ HCl per 100 c.c. and a slightly acid solution of silica was thereby obtained entirely free from common salt. To this solution the requisite quantity of nutrient mineral salts was added to give a concentration of 1 grm. AmCl, 0·2 grm. $MgSO_4$, 0·5 grm. K_2HPO_4 and a trace of $FeCl_3$ per 1,000 c.c., the medium was placed in plugged tubes and sterilized in the autoclave at about 10 lb. pressure. The tubes thus prepared remained fluid for about a month.

When the sterile tubes of silica solution were required for plating purposes, they were first of all inoculated in the usual way and poured into sterile Petri dishes. To each dish was then added a quantity of sterile Na_2CO_3 solution sufficient to neutralize the free HCl present and the dish gently tilted so as thoroughly to mix the contents. The silica solution at once sets to a jelly so that the plates can be conveniently manipulated.

The methods adopted for the preparation of the crude cultures have been described in a previous publication[2] and these were plated out in the manner described and incubated in an atmosphere consisting of a mixture of methane and oxygen. Very small colonies developed at the end of 5-6 days which when placed in Botkin culture flasks containing mineral nutrient solution and filled with a mixture of methane and oxygen gave rise to a strong growth and brought about the oxidation of the former gas.

Description of the bacterium.

The bacteria thus isolated were found to consist of a single species which grew quite well on all ordinary media and also on mineral agar, but in all these

[1] Stevens and Temple. *Centl. Bakt.*, Vol. XXI, p. 84.
[2] Harrison and Subramania Aiyer. *loc. cit.*

cases the growths obtained could not be induced to develop their specific reaction when inoculated into mineral nutrient solution entirely free from organic matter. The growth produced by inoculation from these cultures was very poor, but the introduction of *small traces* of peptone, starch, mannitol, gelatine, glucose, lactamide or sodium asparaginate to the mineral solution led to a strong growth without however causing more than a slight oxidation of the methane. Cultures of this type when plated out on silica jelly yielded no result and it became evident that the bacterium when in pure culture after contact with nutrient organic matter loses all power of oxidizing methane. Consequently it was impossible to complete the investigation by isolating in purely mineral media, transferring through organic media, and finally isolating again on mineral media before determining the oxidizing effect.

In these circumstances to make certain that the bacterium which oxidizes methane in a purely mineral environment is the same as the one whose cultural characteristics on organic media are herein described, was only possible by greatly increasing the number of definite and distinct observations. To this end many crude cultures have been prepared from different rice soils at different seasons of the year and plated out on silica plates. Well isolated colonies have been taken from the plates, tested for their power of oxidizing methane and studied when growing both on mineral and organic media. In all cases the results obtained have been identical and the bacteria isolated on mineral plates have always developed the same characteristics when grown on organic media and have then always lost the power of oxidizing methane. There can, therefore, be no reasonable doubt, but that we are dealing with one and the same bacterium.

The organism is an actively motile bacterium 2—4 μ long and 0·5 μ wide possessing a single (more rarely two) polar flagellum and is therefore classed as a pseudomonas. It is easily stained by all ordinary staining fluid but is *decolourized* by Gram's method. The cultural characteristics are as follows :—The surface colonies on agar are white, opalescent and spreading, but appear small and roundish in the depth. The surface gelatine colonies are slightly sunken owing to liquefaction taking place and in addition there is at first a local greenish-brown coloration which later permeates the whole mass of the gelatine producing a greenish fluorescence. This coloration is characteristic of the cultures on most organic media.

Gelatine stabs are characterized by a faint growth along the line of puncture and, in the course of 48 hours, the gelatine becomes liquefied with a slight sediment at the bottom of the liquid portion. The liquefaction is at first crateriform, then saccate, and finally it extends to the side of the tube and the greenish fluorescence becomes well developed. Agar streak cultures are at

first white and slimy but develop the greenish tint later and finally become brownish. The growth on potato is at first slight and brownish but afterwards spreads until the whole of the potato is discoloured. Bouillon is cloudy at first with a pellicle but the greenish tint develops later and no indol could be detected. There is a strong evolution of gas in nitrate bouillon and nitrate peptone in the course of 24 hours, whilst milk is peptonized and the surface covered with a pellicle. The colour of litmus milk is discharged. Slight acidity is produced in glucose, sucrose and lactose bouillon and the colour of the litmus is discharged.

These cultural characteristics agree entirely with those for *B. Fluorescens liquefaciens* as described by Lehmann and Neuman, and it is interesting to note that Söhngen[1] has shown this bacterium to be capable of oxidizing such hydrocarbons as benzene and paraffin and using them as an energy source when grown in a liquid mineral medium containing 1 per cent of hydrocarbon.

The oxidation of methane.

The method employed in measuring the oxidizing power of this bacterium was essentially the same as that used in similar experiments on the oxidation of hydrogen[2], the apparatus being shown in the annexed sketch.

The neck of a bottle is closed with a rubber cork through which a three-way glass stop-cock C passes, the exit tubes D and E of which are plugged with sterile cotton wool. About 30 c.c. of the nutrient solution are placed in the bottle, the cork and stop-cock inserted and the whole sterilized in the autoclave. The liquid is inoculated by momentarily removing the cork and the exit tube D is then connected to an air-pump and the inlet tube E to a reservoir containing the mixed gases experimented with.

The air is pumped out of the bottle until the liquid boils and gas is then admitted through E from the reservoir. This process is repeated three to four times until it is perfectly certain that the bottle is filled with the mixed gas and that there is no admixture with air.

After inoculation the bottles are first weighed and then stored under water and incubated at

[1] Söhngen. *Centl. Bakt.*, II Abt., XXXVII, pp. 595—609.

[2] Harrison and Subramania Aiyer. *Mem. Dep. Agri., India, Chem. Series*, Vol. IV, No. 4.

30°C. for about a fortnight. At the end of this period the bottle is wiped dry and again weighed so as to determine whether or not leakage has occurred. The tap C is then opened under water to allow the internal pressure to adjust itself and after bringing the surfaces of the internal liquid and external water to the same level, the tap C is closed, and the bottle wiped dry and again weighed.

Any difference in weight is equivalent to an alteration in the volume of the contained gas which is reduced to N. T. & P. both before and after the experiment. Finally the remaining gas is removed and analysed and any changes in composition which it has undergone noted. Knowing the composition and volume before and after the experiment it is possible to calculate the alteration in volume of the component gases.

This method has one disadvantage when applied in this particular instance in that the solubility of CO_2 in water causes the volume of the gas obtained after the reaction to be too low. This, however, does not affect materially the determination of the other gases present so that the disappearance of oxygen and methane is the real measure of the change.

A few typical experiments are quoted to show the kind of result obtained when under autotrophic conditions.

TABLE I.

Showing action under autotrophic conditions.

c. c. N. T. & P.

EXPERIMENT	No. I			No. II			No. III		
DAYS INCUBATION	14 DAYS			21 DAYS			8 MONTHS		
	Before	After	Diff.	Before	After	Diff.	Before	After	Diff.
CO_2 ...	1·7	9·0	+7·3	...	5·5	+5·5	...	7·5	+ 7·5
O ...	86·1	59·2	−26·9	57·7	39·4	−18·3	59·1	29·4	−29·7
CH_4 ...	33·8	18·5	−15·3	29 4	19·1	−10·3	30·1	14·4	−15·7
N ...	26·9	27·6	+0·7	55·0	55·0	..	56·4	58·6	+ 2·2
TOTAL ...	148·5	114·3	−34·2	142·1	119·0	−23·1	145 6	109·9	−35·7

Considering the great variation in period of incubation between these experiments, it is evident that the reaction in pure culture does not reach completion, and it would appear probable that this is only attained under natural condition. It is also noticeable that the ratios $\frac{CH_4 \text{ Disappearing}}{O \text{ Disappearing}}$, *i.e.*,

1/1·75, 1/2·06, 1/1·89, respectively, are not far removed from the theoretical ratio ½ required by the equation :—

$$CH_4 + 2O_2 = CO_2 + 2H_2O.$$
2 Vols. 4 Vols. = 2 Vols.

Hence it would appear that only very small amounts of C are assimilated under the experimental conditions, thus accounting for the non-completion of the reaction and also adding to the probability that this bacterium only reaches its maximum efficiency in mixed culture.

It has been stated previously that after the bacterium was allowed to grow on ordinary media or even on mineral agar, no growth could be induced in mineral nutrient solutions entirely devoid of organic matter. The organism would, however, grow quite vigorously when transferred from organic media to such mineral solutions containing traces of organic matter and experiments to test the CH_4 oxidation were conducted with solutions containing such substances as sodium asparaginate, peptone, starch, mannitol, glucose, lactamide, humus, etc. The results obtained are given in Table II.

TABLE II.

Showing the results obtained in presence of organic matter.

c. c. N. T. & P.

Substance added	0·03 PER CENT. SODIUM ASPARAGINATE			0·1 PER CENT. PEPTONE		
	Before	After	Diff.	Before	After	Diff.
CO	1·8	3·1	+1·3	0·2	3·8	+3·6
O	54·2	51·7	−2·5	60·2	48·9	−11·3
CH_4	33·0	32·3	−0·7	25·1	24·7	−0·4
N	7·9	9·6	+1·7	12·4	13·1	+0·7
TOTAL	96·9	96·7	−0·2	97·9	90·5	−7·4

Substance added	0·03 PER CENT. PEPTONE			0·05 PER CENT. MANNITOL			0·05 PER CENT STARCH		
	Before	After	Diff.	Before	After	Diff.	Before	After	Diff.
CO_2	0·7	1·3	+0·6	2·2	7·9	+5·7	2·0	1·7	−0·3
O	106·7	102·5	−4·2	92·9	84·4	−8·5	85·0	83·1	−1·9
CH_4	54·9	54·3	−0·6	49·4	48·0	−1·4	45·2	45·0	−0·2
N	13·2	14·7	+1·5	10·9	13·0	+2·1	9·9	10·6	+0·7
TOTAL ...	175·5	172·8	−2·7	155·4	153·3	−2·1	142·1	140·4	−1·7

TABLE II.—*Concld.*

Substance added	0·05 PER CENT. HUMUS			0·05 PER CENT. GLUCOSE			MINERALS ONLY		
	Before	After	Diff.	Before	After	Diff.	Before	After	Diff.
CO_2	2·0	1·4	−0·6	2·0	7.2	+5·2	0·2	...	−0·2
O	85·6	83·2	−2·4	86·5	78·5	−8·0	55·2	52·8	−2·4
CH_4	45·5	43·9	−1·6	46·0	45·8	−0·2	29·4	28·5	−0·9
N	10·0	13·9	+3·9	10·2	11·2	+1·0	6·3	6·9	+0·6
TOTAL ...	143·1	142·4	−0·7	144·7	142·7	−2·0	91·1	88·2	−2·9

The organism used in these experiments was obtained from an active culture in mineral media so that it is very evident that contact with organic matter practically inhibits the power of oxidizing methane and particularly in the presence of sugars the main action is one of oxidation of the organic matter in preference to methane. In several instances, however, there is an appreciable oxidation of methane and therefore it seemed probable that a reduction of the amount of organic matter below the quantities used would make this more apparent. The experiment was therefore varied by inoculating from an organic medium into a series of mineral nutrient solutions containing graduated amounts of glucose. The results obtained are shown in Table III.

TABLE III.

Showing effect of bacterium when transferred from an organic medium into solutions of glucose.

c. c. N. T. & P.

Glucose	0·5 PER CENT.			0·1 PER CENT.			0·05 PER CENT.		
	Before	After	Diff.	Before	After	Diff.	Before	After	Diff.
CO_2	...	26·2	+26·2	...	11 0	+11·0	...	7·2	+7·2
O	96·3	66·8	−29·5	92·3	79·1	−13·2	101·1	92·4	−8·7
CH_4	30·2	29·9	−0·3	29·0	29·1	+0·1	31·8	30·9	−0·9
N	23 9	23·9	...	22·9	22·5	−0·4	25·1	25·3	+0·2
TOTAL ...	150·4	146·8	−3·6	144·2	141·7	−2·5	158·0	155·8	−2·2

Glucose	0·025 PER CENT.			0·01 PER CENT.			0 005 PER CENT.		
	Before	After	Diff.	Before	After	Diff.	Before	After	Diff.
CO_2	...	3·1	+3·1	0·2	4·0	+3·8	0·9	1·1	+0·2
O	96·6	92·4	−4·2	52·3	39·7	−12·6	92·5	85·8	−6·7
CH_4	30·4	29·8	−0·6	24·2	19·0	−5·2	28·8	26·7	−2·1
N	24·0	23·7	−0·3	13·5	14·7	+1·2	29·4	30·5	+1·1
TOTAL ...	151·0	149·0	−2·0	90·2	77·4	−12·8	151·6	144·1	−7·5

TABLE III.—*Concld.*

	MINERALS ONLY		
	Before	After	Diff.
CO_2 ...	...	2·2	+2·2
O ...	97·7	94·8	−2·9
CH_4 ...	30·7	29·9	−0·8
N ...	24 3	24·4	+0·1
TOTAL ...	152·7	151·3	−1·4

It will be seen that practically no methane is oxidized, but comparatively large quantities of CO_2 are produced through the oxidation of glucose when the concentration of the latter is above 0·01 per cent., whereas with concentrations of 0·01 per cent. appreciable quantities of methane are oxidized. Below 0·01 per cent. the amount of methane oxidized diminishes, and in mineral solution only the amount becomes negligible. It would, therefore, appear that the power of oxidizing methane is dependent upon the power of any strain of the species to assimilate the carbon contained in the gas and that this in turn depends entirely upon the environment previously encountered.

SUMMARY.

1. The bacterium responsible for the oxidation of methane at the surface of the swamp paddy soils examined has been identified as *B. Fluorescens liquefaciens*.

2. The power of oxidizing considerable proportion of methane is only enjoyed so long as the bacterium is propagated on purely mineral media.

3. In the presence of much organic matter this power is reduced to a minimum and is not regained by further inoculation into purely mineral media. In the presence of traces of certain organic substances, the power is appreciable though low.

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W. H. HARRISON, D. Sc

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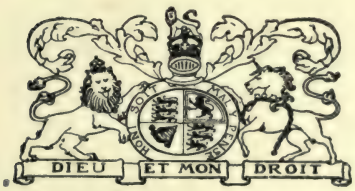

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Imperial Agricultural Chemist.

[Received for publication on 26th April, 1920.]

INTRODUCTORY.

THE study of the composition of paddy soil gases has demonstrated the fact that the main constituents are methane and nitrogen, and that carbon dioxide and hydrogen are found only in comparatively small quantities.[1] The proportion of hydrogen varies from nil to 12 per cent. but, in general, the value does not rise above 3 per cent. in soils carrying a crop, and is usually nil. The values are usually higher in uncropped soils than in cropped ones but the reason for this has been fully discussed in a previous publication.[2]

The proportion of carbon dioxide is also subject to wide variations but it is generally higher than that of hydrogen and is never non-existent. The values vary from about 2 per cent. up to, very occasionally, 20 per cent. but the average in cropped soils is about 5 per cent.

The gases which accumulate in paddy soils are mainly derived from the decomposition of organic matter present in the soil under anaerobic conditions by bacterial agencies.[3] Practically all known decompositions of this type in pure culture are accompanied by a considerable evolution of carbon dioxide and hydrogen, and, except in the presence of nitrates and nitrites, the disengagement of nitrogen is inconsiderable. Even the fermentations resulting in the

[1] Harrison and Subramania Aiyer. *Mem. Dept. Agri., India, Chemical Series*, Vol. III, No. 3.
[2] *Ibid*, Vol. IV, No. 1.
[3] *Ibid*, Vol. III, No. 3, and Vol. V, No. 1.

formation of methane have been shown to include the production of considerable proportions of carbon dioxide and hydrogen. This being the case it is a remarkable fact that paddy soil gases should contain such large proportions of CH_4 and N and such very small proportion of CO_2 and H. In the case of a fairly soluble gas like CO_2 this could possibly be accounted for by solution and consequent removal by the water draining through the soil, but this explanation would be very unsatisfactory when applied to the case of hydrogen. It is also very difficult to account for the presence of such a large proportion of nitrogen in view of the facts that the soil conditions preclude the formation of nitrates or nitrites, at least during the cultivation season, and that the source of much of this N has been shown to be the organic matter present in, or incorporated with, the soil.[1]

Consequently, to account for the observed facts it is necessary to assume either (1) that the fermentation which takes place in paddy soils is of a different type to that generally observed with anaerobic fermentations and one which results in the production mainly of CH_4 and N with practically no CO_2 and H, or (2) that the fermentation is of a normal type, but that certain products, *e.g.*, CO_2 and H, are removed by secondary reactions leaving a residuum of CH_4 and N.

In regard to the accumulation of the considerable volume of nitrogen gas which has been demonstrated in these soils the latter alternative offers a very feasible explanation. No fermentation by pure bacterial cultures, with the exception of those connected with denitrification processes, has been shown to lead to the liberation of sufficient N to account for this fact, but, on the other hand, the presence of small quantities of N in the products has been observed frequently. Given then an ordinary mixed type of anaerobic fermentation combined with removal of the CO_2 and H by some secondary reaction, a residuum would be obtained containing mainly CH_4 and N. In cropped soils it has been shown that the CH_4 is gradually removed by oxidation process and the residual gas would therefore contain a very large proportion of nitrogen commensurate with that actually found in the gases obtained from cropped soils.

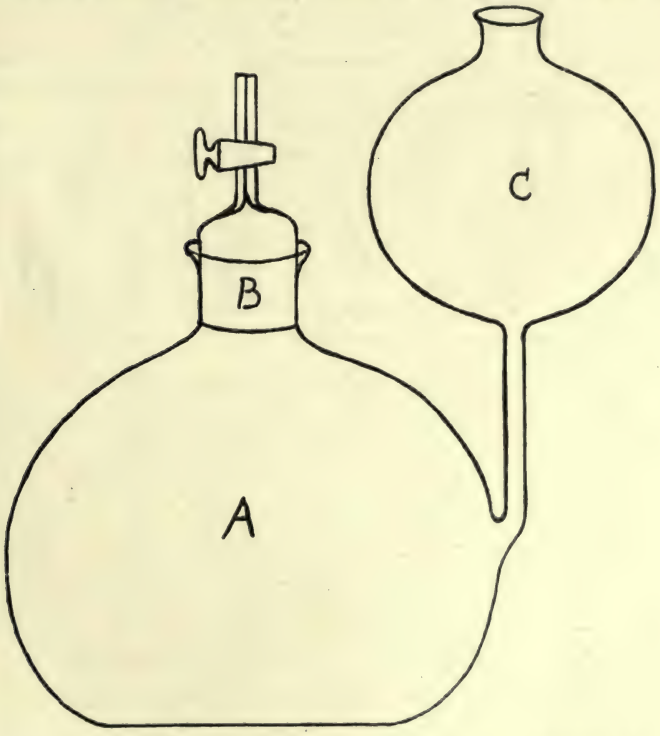
For the purpose of this investigation it was therefore necessary in the first instance to study the fermentation of green-leaf manure.

[1] Harrison and Subramania Aiyer. *Mem. Dept. Agri., India, Chemical Series*, Vol. V, No. 1.

PART I.

THE TYPE OF FERMENTATION OF ORGANIC MATTER INDUCED IN PADDY SOILS AND ITS GASEOUS PRODUCTS.

In order to study the composition of the gases produced by the anaerobic fermentation of green-leaf manure the apparatus shown in the annexed sketch was employed, consisting of a flask A provided with a hollow ground-glass stopper B which terminates in a capillary exit tube closed with a glass tap. From the side of A a side-tube emerges and connects to the spherical reservoir C provided with a small open mouth closed by a plug of cotton wool. The



materials employed in the experiment were introduced into A, which was almost filled with water, and the whole sterilized.. After inoculation sterile water was poured into C until A was completely filled and all air bubbles displaced. As a further precaution to ensure anaerobic conditions the surface of

the liquid in C was covered with a layer of kerosine. Any gas produced during fermentation accumulates in A and can be drawn off for examination at interval through the tap B without introducing air or disturbing the contents of the flask.

In the first series of experiments leaves of *Calotropis gigantia* were placed in the flask, together with the necessary water and, after sterilization in an autoclave, were inoculated by the addition of an emulsion of paddy soil, and the flask completely filled with water. The sterilization of the leaves ensured that no air remained in them. These experiments were repeated many times during a period extending over several years with uniform results and, consequently, it is only necessary to quote one series of results by way of illustration.

TABLE I.

Showing the composition of the gases obtained from sterile green-leaf manure inoculated with paddy soil.

Days	Volume of gas obtained c.c.	Rate of gas evolution c.c. per day	Percentage composition			
			CO_2	H	CH_4	N
0–1	16·8	16·8	34·5	52·4	0·6	12·5
1–14	9·6	0·74	59 4	6·2	24·0	10·4
14–16	12·4	6·2	61·3	4·8	28·2	5·7
16–35	100·0	5·3	48·8	3·4	47·8	...
35–52	87·0	5·0	46·3	2·4	51·0	0·3
52–56	41·9	10·4	31·3	7·6	61·1	...

Thus, immediately, there is induced a rapid fermentation producing mainly CO_2 and H which, however, quickly subsides, and then the rate of gas production remains low for a considerable period of time. Subsequently a fairly rapid fermentation sets in producing mainly CO_2 and CH_4, the hydrogen being small in quantity and N practically non-existent. In the early stage the gas produced contains a relatively high proportion of nitrogen. This sequence of events was not only observed in all experiments of a similar character, but similar results were also obtained when inoculations were made into sterile leaves from flasks in active fermentation instead of using soil direct.

Experiments carried out with sterile leaves do not, however, approximate to field conditions and, consequently, a series was carried out with natural *unsterilized* leaves and soil. It was at once observed that the resulting fermentation was more intense but, notwithstanding this, the type—judged by the composition of the gases—remained the same and neither did the inclusion of fresh paddy roots produce any modification.

TABLE II.

Showing composition of gases obtained from unsterilized green-leaf manure inoculated with paddy soil.

Days	Volume of gas in c.c.	Rate of evolution per day	Percentage composition			
			CO_2	H	CH_4	N
0—1	421	421	76·0	5·8	0·0	18·2
1—2	398	398	96·5	...	0·0	3·5
2—4	141	72	94·2	0·2	3·5	2·1
4—5	125	125	88·1	...	5·4	6·5
5—7	122·7	61	86·5	0·4	12·4	0·7
7—13	97	16	49·5	...	50·5	0·0

The increased intensity of the fermentation when using unsterilized leaf is not due to enzymic action as shown by the fact that the addition of toluene, etc., entirely inhibits gas production. In addition, inoculation from a flask in active fermentation into *sterile* green-manure reproduces the fermentation.

TABLE III.

Composition of gases obtained by inoculation of sterile green-leaf manure from an active fermentation.

Days	Volume of gas c.c.	Rate	Percentage composition			
			CO_2	H	CH_4	N
0—3	27·9	9·3	95·0	.....	.....	5·0
3—5	20·6	10·3	96·2	.....	.....	3·8
5—6	19·9	19·9	93·0	.....	4·0	3·0
6—7	30·6	30·6	81·4	3·7	8·8	6·1
7—8	37·0	37·0	84·4	1·8	13·2	0·6
8—9	19·0	19·0	82·2	2·1	14·7	1·0
9—10	19·4	19·4	79·9	2·6	16·5	1·0
10—13	74·8	24·9	63·6	3·1	23·1	10·2
13—14	51·4	51·4	61·2	10·9	28·0	trace
14—15	44·3	44·3	54·8	... 45·4	.....	nil
15—17	11·4	5·7	37·7	0·0	62·3	0
17—19	33·2	16·6	23·5	0·0	76·5	0
19—21	52·0	26·0	20·0	5·5	74·5	0
21—23	22·0	11·0	14·5	0·3	85·2	0
23—24	44·3	44·3	16·9	4·5	78·6	0
24—26	49·0	24·5	15·7	17·5	66·8	0

These fermentations take place under very artificial conditions and it is very probable that under natural conditions the proportion of the disengaged gases varies from those obtained. Nevertheless, it appears to be permissible to draw the following general conclusions :—

(*a*) The addition of green-leaf manure to paddy soils gives rise to a strong fermentation resulting in the liberation of CO_2 and H and N

(*b*) This fermentation rapidly decreases in intensity and is replaced by a fermentation which liberates mainly CO_2 and CH_4.

(*c*) The fermentation is of a bacterial nature as shown by the fact that it is inhibited by the presence of toluene.

It was easily demonstrated that the first fermentation is due to specific bacteria by plating out on nutrient agar and inoculating the colonies into tubes of sterile green-leaf manure provided with gas tubes. A strong fermentation was induced by bacteria belonging to two distinct types.

Type I. Motile, sporing bacteria stained by Gram's method and which liquefy gelatine. Strong growth on potato with, occasionally, the production of gas ; acid produced but no gas in glucose and lactose media ; milk slightly acid but no coagulation ; nitrates not reduced and indol not produced.

Type II are non-motile, non-sporing bacteria unstained by Gram's stain and give a dry, crumpled brown growth on potato. Indol produced.

Type I induced a very active fermentation when inoculated into flasks containing green-leaf manure, the gases produced consisting mainly of CO_2 and H together with appreciable quantities of N. Type II produces a much less intense fermentation and the proportion of N liberated is higher. Both fermentations cease within 96 hours.

TABLE IV.

Showing volume and composition of gases produced by type bacteria under similar conditions.

Type	Volume of gases in 96 hours	PERCENTAGE COMPOSITION			
		CO_2	H	CH_4	N
I a	288	27·9	67·7	...	4·4
I b	192	22·5	75·2	..	2·3
II a	83	16·7	65·3	...	18·0
II b	46	23·5	68·2	...	6·7

These organisms are closely related so far as their activities lie in bringing about of fermentation of green-manure. They induce a rapid fermentation resulting in the production of CO_2 and H together with some nitrogen, but they do not bring about a disintegration of the leaf and appear to confine their activities to intercellular substances and their action soon ceases. They may be looked upon as being responsible for the first stage of the decomposition of green-manures in paddy soils and to pave the way for the main fermentation, which is associated with the production of methane.

This second fermentation occurs, *i.e.*, the methane fermentation, when the residuum from the first fermentation is allowed to stand for some time, but is much more rapidly induced if this residuum is sterilized and then re-inoculated with fresh paddy soil. Thus within six days from the initiation of the fermentation 162 c.c. of gas were collected, containing 30·6 per cent. CO_2, 4·5 per cent. H, 60·4 per cent. CH_4 and 4·5 per cent. N.

Considerable difficulty was experienced in establishing this fermentation in synthetic media but, ultimately, active cultures were obtained in solutions of calcium butyrate and lactate and also with filter paper. In all cases the gas produced had a similar composition to that shown above.

PART II.

THE RELATIONSHIP OF CO_2 AND H TO PADDY SOILS.

The study of the fermentation of green-leaf manure shows that under anaerobic conditions such as obtain in swamp paddy soils there is the production of considerable volumes of CO_2, H, CH_4 and only a small proportion of N, whereas the gases which accumulate in the soil have been shown to consist mainly of N and CH_4 with practically no CO_2 and H and, in fact, in cropped soils the N preponderates.

The disappearance of the large volumes of CO_2 and H may be explained (*a*) by solution of the CO_2 in, and removal with, the water draining through the soil and (*b*) by the oxidation of the H in a manner analogous to that of the CH_4 by the surface film, but, even if these explanations are accepted, it is difficult to reconcile them with the low values found for these substances in the soil gases. Some other additional explanation is necessary and the only feasible one appeared to be that the CO_2 and H are recombined under the action of agencies existing in the soil, or that the reduction of the CO_2 by H provided energy for bacterial activities.

To test this hypothesis 10 grams of soil and 25 c.c. of water were sealed up in tubes containing a mixture of CO_2 and H. At the end of seven days the tubes were opened over mercury and the residual gases measured and analysed :—

TABLE V.

Showing disappearance of CO_2 and N when sealed up with paddy soil and water.

c.c. N. T. & P.

Constituent	Experiment I			Experiment II		
	Before	After	Diff.	Before	After	Diff.
CO_2	51·4	29·0	−22·4	205	105	−100
H	251·0	65·2	−185·8	131	31·7	−99·3

There was a strong bacterial development in the tube and it was found possible to carry the reaction through a series of transfer inoculated into the following solution :—

	Per cent.
K_2HPO_4	0·05
$MgSO_4$	0·02

	Per cent.
NH_4Cl	0·10
$NaHCO_3$	0·05

traces of $CaSO_4$, $MnCl_2$ and $FeCl_3$

In all cases the production of large proportion of sulphuretted hydrogen was noticeable and consequently, under the experimental conditions, much of the loss of hydrogen was due to sulphate reduction.

TABLE VI.

Showing the production of sulphuretted hydrogen.

c. c. N. T. & P.

Constituent	Before	After	Diff.
CO_2	151·5	41·4	−72 5
H	182·0	100·8	−81·2
SH_2	nil	73·61	+73·61

A parallel series of experiments carried out with CO_2 only, and also with a mixture of CO_2 and CH_4, gave no indication of absorption and, consequently, the reaction only takes place in the presence of hydrogen. This would show that CO_2 and H cannot persist in admixture in paddy soils and that their disappearance is bound up with the metabolism of the sulphur bacteria.

The presence of sulphate is, however, not essential to the reaction as is shown by inoculation and transfer inoculations into the following solution which is devoid of sulphate :—

	Per cent.
K_2HPO_4	0·05
$MgCl_2$	0·02
NH_4Cl	0·10
$NaHCO_3$	0·05

traces of $CaCl_2$, $MnCl_2$ and Fe_2Cl_6

TABLE VII.

Showing disappearance of CO_2 and H in the absence of sulphates.

c. c. N. T. & P.

Constituent	Experiment I, crude culture 4 days' inoculation			Experiment II. crude culture 9 days' inoculation		
	Before	After	Diff.	Before	After	Diff.
CO_2	52·2	31·1	−21·1	47 8	7·6	−40·2
H	89·6	63·6	−26·0	81 9	nil	−81·9
N	4·6	4·6	0·0	4·1	5·2	+1·1

The disappearance of the CO_2 and H is, therefore, not necessarily associated with sulphate reduction but is also due to reduction of the CO_2 and on the assumption that the carbon is assimilated by the organisms the reaction would form an energy source :—

$2H_2+CO_2+aq=C+2H_2O+aq.....+34$ Cals. In this reaction the ratio $\frac{\text{Vol } CO^2}{\text{Vol. H}}=\frac{1}{2}$ and consequently the measurement of the ratio between the disappearing gases will give some idea of the correctness of this view.

Determination of Ratio $\frac{CO_2}{H}$. In Table VII the ratios for experiments I and II are $\frac{1}{1\cdot3}$ and $\frac{1}{2\cdot0}$ respectively, and show no agreement. In fact from a large number of experiments carried out it was found that the ratio was far from constant and values varying up to almost $\frac{1}{4}$ were obtained. The early experiments were carried out in the manner described in a previous publication,[1] and the variations were at first ascribed to errors of experiment, but 'blank' tests carried out showed that the method of experiment was accurate to less than 2 per cent. Consequently some other explanation must be sought for. This was found in the fact that, coincident with the disappearance of CO_2 and H_2, there were formed varying proportions of CH_4, and that when the proportion of the latter was low the ratio was under $\frac{1}{2}$, and that where it was high the ratio was about $\frac{1}{3}$ to under $\frac{1}{4}$.

Three examples are given in the following Table :—

TABLE VIII.

Showing variations in the ratio $\frac{CO_2}{H}$.

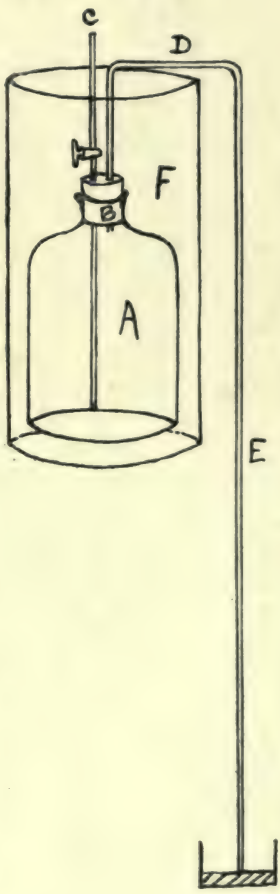
c. c. N. T. & P.

Constituent	Experiment I			Experiment II			Experiment III		
	Before	After	Diff.	Before	After	Diff.	Before	After	Diff.
CO_2	60·4	45·4	−15·0	123·7	93·8	−29·9	121·7	102 0	−19·7
H	73 4	49·4	−24·0	68·2	12·1	−56·1	67·1	nil	−67·1
CH_4	nil	nil	nil	nil	3·5	+3·5	nil	12·7	+12·7
N	4·9	4·3	−0 6	4·2	7·5	+3·3	3·9	5·4	+1·5
Ratio $\frac{CO_2}{H}$	Ratio =		$\frac{1}{1\cdot6}$	Ratio =		$\frac{1}{1\cdot9}$	Ratio =		$\frac{1}{3\cdot5}$

[1] Harrison and Subramania Aiyer. *Mem. Dept. Agri., India. Chemical Series*, Vol. IV, No. 4.

It is therefore evident that the reaction which leads to the disappearance of the CO_2 and H is one of reduction of the former, and it is also evident that it is not a simple reaction, but rather one compounded of two distinct reactions, *e.g.*, one resulting solely in the formation of organic matter and the other resulting in the formation of organic matter together with the production of CH_4.

The rate at which the mixed gas disappears. With the later experiments a method was adopted which permitted the changes in volume to be followed continuously. The apparatus used is shown in the annexed sketch.



A few c.c. of an active culture, or 1 gram of soil, and a few c.c. of the mineral nutrient solution are introduced into the wide-mouthed bottle A which is closed with the india-rubber stopper B carrying two glass tubes C and D. The tube C is closed with a stop-cock and serves as the entry-tube for the mixed gas. The exit tube D is connected by means of a mercury sealed rubber joint to a longer piece of tube E which dips into a basin of mercury and serves as a manometer. The mixture of gases is passed through the bottle A until all air is displaced and the stop-cock then closed, but as the gas in A is under excess pressure equilibrium with the external air is obtained by momentarily opening and closing the stop-cock. The bottle is immersed in a larger vessel F filled with water which was generally surrounded by a thick felt jacket in order to exclude the light. As the reaction progressed the mercury rose in the tube E and the height is a measure of the change in volume of the gases in A. The residual gases were removed by running mercury into A by the tube C and then analysed. The following Table gives some of the results obtained taking the original volume as 100 in each case—all values being corrected to N. T. and P.

TABLE IX.

Day	EXPERIMENT I Paddy soil in the light	EXPERIMENT II. Paddy soil in the dark	EXPERIMENT III Made from active culture in the dark
0	100	100	100
1st	...	98·9	98·6
2nd	...	98·1	96·0
3rd	97·6	97·0	94·2
4th	97·6	...	89·1
5th	97·5	...	80·8
6th	...	95·7	69·0
7th	...	...	58·4
8th	...	93·0	47·8
9th	97·1	92·2	44·0
10th	...	91·0	44·0
11th	96·0	87·2	44·0
		Refilled with gas mixture	
12th	...	84·1	100
13th	93·8	79·2	98·0
14th	...	65·2	91·7
15th	90·5	...	85·8
16th	...	56·8	72·2
17th	88·9	49·3	65·8
18th	...	45·4	59·8
19th	87·0	44·7	53·0
20th	...	44·5	46·8
21st	83·2	44·5	43·1
22nd	81·0	44·5	42·7
30th	72·3		
40th	61·9		
50th	54·5		
63rd	46·0		

In all the above experiments the gaseous mixture was approximately 50 per cent. CO_2 and 50 per cent. H and 'blank' sterile experiments were also carried out which showed no absorption. All three experiments agree in showing that the absorption is small until the growth is well established and then the rate of absorption is extremely rapid. The effect of light in retarding the reaction is also well brought out in Experiment I and it is also clearly shown that inoculation from an active culture establishes the reaction quicker than does inoculation with paddy soil.

It is quite clear that once the reaction is started it proceeds at a fairly uniform rate until it suddenly ceases. On again passing a fresh supply of gas into the bottle the reaction recommences and proceeds as before and again ceases when the same degree of absorption has approximately been reached. This procedure can be repeated several times with similar results and indicates clearly that the exhaustion of one or other of the constituent gases determines the point at which absorption ceases.

SUMMARY AND GENERAL CONCLUSIONS.

1. No evidence has been obtained to show that the fermentation of green-manure under anaerobic conditions takes an abnormal or unusual course. The gaseous products of this fermentation appear to be carbon dioxide, hydrogen, methane, and a small proportion of nitrogen.

2. The absence of the CO_2 and H from paddy soil gases is accounted for by the fact that a subsidiary bacterial action takes place resulting in the reduction of the CO_2 by the hydrogen and the formation of organic matter and even marsh gas. *CO_2 and H cannot as a matter of fact persist in association in paddy soils.*

3. The proportion of CO_2 to H which disappears in this reaction varies from 1 : 1·5 to 1 : 3·5 according to the proportion of marsh gas produced, so that much more H disappears than CO_2. On the other hand, the evidence obtained in this investigation goes to show that more CO_2 is produced than H, so that one would naturally expect more CO_2 than H to be generally present in paddy soil gases. This is in accordance with previously observed facts.

4. This reaction between CO_2 and H is beneficial to the paddy crop in several ways. In the first place, the amount of free H in the soil gases is kept at a very low value and consequently the amount of this gas which reaches the surface film is negligible. It has been shown previously that the surface film is capable of oxidizing H, and if large quantities of H reached it they would be oxidized at the expense of oxygen which would otherwise be available for root aeration. On the other hand, any excess of CO_2 in the soil gases would be decomposed with the liberation of oxygen immediately it came in contact with the surface film. *The reaction is, therefore, an important factor in the conservation of the oxygen available for root aeration.*

In the second place, the formation of marsh gas is of extreme importance by increasing the supply of that substance to the surface film and thereby also increasing the oxygen supply in the manner indicated in a previous publication. It is also permissible to assume that the organic matter also produced during the reaction is sooner or later subjected to a similar fermentation with similar results, so that there is a complicated carbon cycle in these soils which results in the conservation of the carbon and ensures the maximum amount being converted into methane with a correspondingly increased efficiency of root aeration. *This reaction is, therefore, mainly concerned with the conservation of carbonaceous matter and its utilization so as to produce the maximum amount of root aeration.*

Thirdly, by keeping the carbon dioxide content of the soil gases at a minimum the efficiency of the aeration of the roots is proportionately increased.

Finally, this reaction permits of a rational explanation of the liberation of nitrogen gas without having to call in the aid of a peculiar and undemonstrated fermentation of nitrogenous organic matter. The facts now put forward show that there is taking place continually in these soils a resolution of organic matter followed by a recombination and at each turn of the cycle small quantities of nitrogen are liberated. In the aggregate, during a cropping season, the volume of nitrogen thus liberated will assume large proportions, and consequently *the liberation of gaseous nitrogen in paddy soils is the resultant of the complicated carbon cycle and not of any specific bacterial fermentation.*

5. This reaction has been studied in relation to paddy soils, but it would appear to be by no means specific to such soils. The same reaction has been obtained when a Pusa soil, which has never been subjected to wet cultivation, was substituted for the paddy soil in the experiments. It would, therefore, appear probable that the active agents producing this reaction are widely diffused and become operative when the conditions become favourable. In effect, much which has been ascribed to a specific methane fermentation, *e.g.*, in marsh land, sewage, septic tanks, etc., would rather appear to be due to this reaction.

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